REMARKS/ARGUMENTS

Reconsideration is requested. Claims 1-16, 18 and 20-25 are pending.

I. THE 35 U.S.C. §112, SECOND PARAGRAPH, REJECTION

Claims 1-25 are rejected under 35 U.S.C. 112, second paragraph, as allegedly indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. The action asserts that it is not clear what is meant by "an analog or derivative", and that in the absence of any way of clearly and distinctly defining the boundaries of this class of compounds, the claims are indefinite. The rejection is respectfully traversed.

Beginning on page 21 of the specification, there are provided definitions of terminology used in the specification and claims. The term "acyl derivative" is clearly defined at page 21, second complete paragraph, and the term "analog" is defined by the entire text on page 22. Based on this, the claims are clearly not indefinite. Withdrawal of the rejection is respectfully requested.

II. THE 35 U.S.C. §112, FIRST PARAGRAPH, REJECTIONS

Claims 1-25 are rejected under 35 U.S.C. §112, first paragraph, because the specification, while being enabling for a method of treating, reducing, or ameliorating the toxicity of a pyrimidine analog, allegedly does not reasonably provide enablement for preventing said toxicity. In response, without conceding to this rejection, the term "preventing" has been canceled without prejudice from claim 1. Withdrawal of the rejection is respectfully requested.

Claims 18, 20, 22, 24, and 25 are rejected under 35 U.S.C. §112, first paragraph, because the specification, while being enabling for methods involving certain specific uridine phosphorylase inhibitors, cytidine deaminase inhibitors, nucleoside transport inhibitors, enhancers of hematopoiesis, and enhancers of uptake and phosphorylation of nucleosides, such as those recited in instant claims 19, 21, and 23, allegedly does not reasonably provide enablement for methods involving administering all possible compounds of these types. In response, and without conceding to this rejection, claim 18 has been amended to incorporate the subject matter of claim 19 and claim 19 has been canceled without prejudice. Claim 20 has been amended to remove the reference to deoxycytidine, and claims 22, 24 and 25 are each directly dependent on claim 1 which is not rejected on lack of enablement grounds. Withdrawal of the rejection is respectfully requested.

III. THE OBVIOUSNESS REJECTIONS

Claims 1-5, 8, 10 and 11 are rejected under 35 U.S.C. §103(a) as allegedly unpatentable over Kawaguchi *et al.* '139 (US patent 4,757,139, cited in PTO-892) or the equivalent to PCT publication W085/00608, published Feb. 14, 1985). The Action asserts that Kawaguchi '139 discloses an acylated pyrimidine nucleoside analog, specifically an acylated 5-fluoro-2'-deoxyuridine (column 2 lines 15-38), and that this compound shows strong antitumor activity even in low doses and has a markedly improved therapeutic index over the parent compound 5-fluoro-2'-deoxyuridine (column 2 lines 39-48). The Action asserts that while Kawaguchi '139 does not disclose a method whereby these acylated compounds are substituted for 5-fluoro-2'-deoxyuridine

in order to prevent or treat toxicity due to this compound, it would allegedly have been obvious to one of ordinary skill in the art at the time of the invention to prevent or treat toxicity due to 5-fluoro-2'-deoxyuridine by administering the controlled release esters of Kawaguchi '139 instead of unmodified 5-fluoro-2'-deoxyuridine. This rejection is respectfully traversed.

As now claimed, the method of the invention is directed to a method for treating toxicity due to a pyrimidine nucleoside analog comprising administering to an animal a pharmaceutically effective amount of an acylated derivative of uridine or cytidine, for example triacetyluridine. Kawaguchi '139 does not disclose or suggest the use of acylated compounds of uridine and cytidine (naturally occurring nucleosides – see page 21 of the application) in order to prevent or treat toxicity due to 5-fluoro-2'-deoxyuridine. The disclosure in Kawaguchi '139 of an acylated 5-fluoro-2'-deoxyuridine clearly would not have motivated one of ordinary skill to use an acylated derivative of uridine or cytidine for treating toxicity due to a pyrimidine nucleoside analog. Withdrawal of this rejection is respectfully requested.

Claims 1-5, 8, 10, 11 and 17 are rejected under 35 U.S.C. §103(a) as allegedly unpatentable over Takai *et al.* (Foreign Patent JP-560-126221, reference included with PTO-892). The Action asserts that Takai discloses an antitumor composition comprising 5-fluoro-2'-deoxyuridine (I) and a thymidine compound that can be an acylated thymidine (abstract, also p. 140). While Takai does not disclose a method whereby the acylated pyrimidine nucleoside is non-methylated (i.e. uridine), the Action asserts that it would have been obvious to one of ordinary skill in the art at the time of

the invention to substitute acylated uridine for acylated thymidine in the methods of Takai. The rejection is respectfully traversed.

Takai discloses thymidine derivatives or prodrugs for reducing toxicity of 5-fluoro-2'-deoxyuridine prodrugs. Fluorodeoxyuridine and fluouracil (to which a good fraction of administered fluorodeoxyuridine is converted) are cytotoxic against both tumors and normal cells via two major mechanisms of action.

1. Inhibition of thymidylate synthase by fluorodeoxyuridine monophosphate (FdUMP).

Inhibition of this enzyme starves cells of thymidine for DNA synthesis. Relatively low doses of fluorouracil or fluorodeoxyuridine are sufficient to inhibit this enzyme. Thymidine or thymidine prodrugs can reverse toxicity caused via this mechanism, which, however, can reduce antitumor efficacy.

2. Incorporation of fluorouracil into RNA.

When a large dose of fluorouracil (or fluorodeoxyuridine) is administered, it can be converted to fluorouracil triphosphate (FUTP) which can substitute for UTP for RNA synthesis. This mechanism is especially important for toxicity of bolus doses of fluorouracil, and accounts for the sometimes lethal toxicity of fluorouracil overdoses.

However, there as important differences with regard to thymidine or thymidine prodrugs and uridine or uridine prodrugs. First, thymidine or thymidine prodrugs are ineffective against this mechanism of toxicity involving RNA, whereas uridine or compounds that are converted to uridine *in vivo* (uridine or cytidine prodrugs, for example - cytidine is readily converted to uridine) can reduce this toxicity if administered within about 48 hours or so after an otherwise toxic or lethal dose of fluorouracil.

Secondly, thymidine is not incorporated into RNA and, therefore, cannot reduce toxicity due to FUTP incorporation into RNA. On the other hand, uridine does not reverse thymidylate synthase inhibition by FdUMP.

It is been observed that intentional administration of high-dose fluorouracil followed by oral triacetyluridine results in selective protection of normal tissues with enhanced antitumor efficacy (Saif and von Borstel, 2005 - copy attached). However, this strategy does not work with thymidine (or thymidine prodrugs) as the rescue agent. The attached paper by Klubes and Cerna (1983) demonstrates that uridine but not thymidine (dThy) reverses toxicity of high doses of 5FU. Klubes and Cerna did not however address or solve the problem of uridine delivery to humans, which is solved by use of oral triacetyluridine (and other acyl derivatives of cytidine and uridine).

Based on the above, it is clear that one of ordinary skill would not have been motivated to arrive at the present invention based on the Takai disclosure of a 5-fluoro-2'-deoxyuridine/acylated thymidine composition. Withdrawal of this rejection is requested.

The obviousness rejection of claims 18 and 19 under 35 U.S.C. §103(a) as allegedly unpatentable over Kawaguchi '139 and further in view of Chu *et al.* and the obviousness rejection of claims 18 and 19 under 35 U.S.C. §103(a) as allegedly unpatentable over Takai in view of Chu are traversed for the same reasons as urged above in regard to Kawaguchi '139 and Takai. Dependent claims 18 and 19 have been combined, and claim 19 has been canceled without prejudice. Chu does not cure the underlying deficiencies of Kawaguchi and Takai as discussed above. Withdrawal of these rejections is respectfully requested.

Claims 1-6, 8, and 10-12 are rejected under 35 U.S.C. §103(a) as allegedly unpatentable over Kawaguchi *et al.* '162 (US patent 4,868,162). While Kawaguchi '162 discloses an acylated 5-halo-2'-deoxyuridine, such as fluoro-, chloro-, or bromo-nucleosides, there is no disclosure of a method whereby these acylated compounds are substituted for 5-fluoro-2'-deoxyuridine in order to prevent or treat toxicity due to 5-fluoro-2'-deoxyuridine. This rejection is traversed for the same reasons urged above in regard to the obviousness rejection of the claims over Kawaguchi '139. Thus, the invention as claimed is directed to treating toxicity due to a pyrimidine nucleoside analog by administering a pharmaceutically effective amount of an acylated derivative of uridine or cytidine. Kawaguchi '162 does not disclose or suggest the use of acylated compounds of uridine and cytidine (naturally occurring nucleosides) for treating toxicity due to 5-fluoro-2'-deoxyuridine. The skilled artisan would not have been motivated by the Kawaguchi '162 disclosure of an acylated 5-halo-2'-deoxyuridine to use an acylated derivative of uridine or cytidine for treating pyrimidine nucleoside analog-induced toxicity. Withdrawal of this rejection is respectfully requested

IV. DOUBLE PATENTING

Claims 1-10 and 14-25 are rejected on the ground of nonstatutory obviousness-type double patenting as allegedly unpatentable over claims 1-23 of U.S. Patent No. 5,968,914 (the '914 patent). This rejection is traversed.

> A patent issuing on an application with respect to which a requirement for restriction under this section has been made, or on an application filed as a result of such a requirement, shall not be used as a reference either in the Patent and Trademark Office or in the courts against a divisional application or against the original application or any patent issued on

> either of them, if the divisional application is filed before the issuance of the patent on the other application.

35 U.S.C. 121.

The '914 patent issued from application Serial No. 08/472,210 (filed on June 7, 1995) which is a continuation-in-part of application Serial No. 08/176,485 (the '485 application). During prosecution of the '485 application, Examiner Kunz required restriction among the claims (Office Action mailed June 28, 1994 in the '485 application). He found there were three distinct inventions: Group I (claims 1-25 drawn to methods for preventing or treating the toxicity caused by pyrimidine nucleosides), Group II (claims 27-47 drawn to methods for treating cancer), and Group III (claims 75-87 drawn to compositions of acylated pyrimidine nucleosides and a chemotherapeutic agent). The claims of the '914 patent are drawn to methods for treating cancer.

The present application is a divisional of the '485 application filed before the issuance of the '914 patent. The pending claims are drawn to methods for preventing or treating the toxicity caused by pyrimidine nucleosides. Therefore, 35 U.S.C. § 121 prohibits using the '914 patent as a reference against this application.

Moreover, the restriction requirement in the parent '485 application is evidence that the pending claims 1-25 are patentable over claims 1-26 of the '914 patent because methods for preventing or treating the toxicity caused by pyrimidine nucleosides (i.e., the invention claimed in this application) are distinct from methods for treating cancer (i.e., the invention claimed in the '914 patent). Withdrawal of this rejection is requested.

Claims 1-5 and 8-11 are rejected on the ground of nonstatutory obviousness-type double patenting as allegedly unpatentable over claims 3, 4, and 8 of U.S. Patent No.

6,743,782. In response, with the limitation to the use of an acylated derivative of uridine or cytidine for the treatment of toxicity due to a pyrimidine nucleoside analog, it is clear that there is no overlap with claims 3, 4, and 8 of U.S. Patent No. 6,743,782. Withdrawal of this rejection is requested.

Claims 1-5 and 8-11 are rejected on the ground of nonstatutory obviousness-type double patenting as allegedly unpatentable over claims 1, 4, and 5 of U.S. Patent No. 6,103,701. This rejection is traversed.

The Action asserts that claims 1, 4, and 5 of the '701 patent are directed to methods for delivering exogenous deoxyribonucleosides to a subject (e.g. an animal) by administering an acylated non-methylated pyrimidine. Applicants disagree. Claims 1, 4 and 5 of the '701 patent claims a method of enhancing hematopoiesis in an animal in need of such treatment. Withdrawal of this rejection is requested.

Claims 1-5 and 8-11 are rejected on the ground of nonstatutory obviousness-type double patenting as allegedly unpatentable over claims 9-12 and 22-25 of U.S. Patent No. 6,306,834. In response, with the limitation to the use of an acylated derivative of uridine or cytidine for the treatment of toxicity due to a pyrimidine nucleoside analog, it is clear that there is no overlap with claims 9-12 and 22-25 of U.S. Patent No. 6,306,834. Withdrawal of this rejection is requested.

Claims 1-5 and 8-11 are rejected on the ground of nonstatutory obviousness-type double patenting as allegedly unpatentable over claims 1-11 of U.S. Patent No. 6348451. Without conceding to this rejection, Applicants will consider the submission of a Terminal Disclaimer when the claims are otherwise in allowable condition.

Claims 1-5, 8-11, and 14 are rejected on the ground of nonstatutory obviousness-type double patenting as allegedly unpatentable over claims 1-7 of U.S. Patent No. 6,329,350. This rejection is traversed.

As noted above, the present application is a divisional of U.S. Application No. 08/176,485 (the '485 application), now U.S. Patent No. 5,736,531 (the '531 patent). The claims of the '485 application were restricted (June 28, 1994) among three inventions: Group I (drawn to methods for preventing or treating the toxicity caused by pyrimidine nucleosides), Group II (drawn to methods for treating cancer) and Group III (drawn to compositions of acylated pyrimidine nucleosides and a chemotherapeutic agent). The subject matter of Group I is being pursued in the present application. The subject matter of Group II is claimed in U.S. patent 5,968,914 and U.S. patent 6,344,447, and the subject matter of Group III is claimed in U.S. patent 5,736,531. The restriction requirement in the '485 application is evidence that methods for preventing or treating the toxicity caused pyrimidine nucleoside analogs are patentably distinct over: (1) methods for treating cancer; and (2) compositions. The claims of the '350 patent are directed to methods for treating cancer. As seen from the restriction requirement in the '485 application (discussed above), methods for preventing or treating toxicity caused by pyrimidine nucleoside analogs are patentably distinct from methods for treating cancer. Accordingly, the claims of the subject application and the claims of the '350 patent are patentably distinct. Withdrawal of this rejection is requested.

Favorable action is requested.

Respectfully submitted,

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CLINICAL TRIAL REPORT

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5-Fluorouracil dose escalation enabled with PN401 (triacetyluridine): toxicity reduction and increased antitumor activity in mice

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Abstract *Purpose*: PN401, an oral prodrug of uridine yields more bioavailable uridine than oral administration of uridine itself. PN401 may therefore be useful for permitting dose escalation of 5-fluorouracil (5-FU) with consequent improvements in antitumor efficacy. *Experimental design*: Female BALB/c mice (Colon 26 adenocarcinoma) were treated with 5-FU with PN401 to define the MTD, and pharmacokinetic analyses were done. A comparison of 5-FU/PN401 was made to 5-FU/eniluracil (EU) and 5-FU/LV. The best timing of the first dose of PN401 relative to 5-FU was evaluated by administering groups of mice PN401 beginning 2, 24, or 48 h after 5-FU dose. *Results*: The MTD of 5-FU was 100 mg/kg/week whereas the MTD of 5-FU + PN401 was 200 mg/kg/week. A complete response (CR) of 80% and partial response (PR) of 20% was observed with 5-FU (200 mg/kg) + PN401, CR of 40% and PR of 60% with 5-FU (175 mg/kg) + PN401, PR of 10% with 5-FU (150 mg/kg) + PN401 while no response with 5-FU (100 mg/kg) + PN401. Analysis of 5-FU pharmacokinetics displayed nonlinearity as a function of administered dose in mice. In the comparison study, the best response was achieved with PN401 when compared to EU and LV. Mice that did not receive PN401 died by day 12, while other groups were alive at day 31. The proportion of mice surviving was highest in the group which received PN401 at 2 h followed by 24 and 48 h. *Conclusions*: There is a threshold 5-FU dose after which the efficacy is dramatically improved—in mice bearing Colon 26 adenocarcinoma, that threshold is a dose of > 150 mg/kg/week, and the increased efficacy correlates with about a fourfold increase in the AUC of 5-FU. PN401 used to rescue mice from the lethal toxicity of 5-FU entails that PN401 can be used as an antidote even when used up to 48 h after a 5-FU overdose.

Key words 5-Fluorouracil · PN401 · Fluoropyrimidines · Uridine · DPD

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Introduction

Fluorouracil (5-FU) is one of the most commonly used chemotherapeutic agents and constitutes the mainstay of chemotherapy for most gastrointestinal tumors as well as others [1]. The cytotoxicity of 5-FU involves (1) inhibition of thymidylate synthase, principally via the actions of its metabolite, fluorodeoxyuridine monophosphate (FdUMP) and (2) synthesis of defective RNA as a result of incorporation of a second metabolite, fluorouridine triphosphate (FUTP), into RNA [2]. The principal toxicities of 5-FU include neutropenia, mucositis, diarrhea, and hand–foot syndrome, with the latter two adverse effects predominating when 5-FU is administered as a continuous intravenous (IV) infusion [3]. Like other conventional cytotoxic antineoplastic agents, 5-FU has a relatively narrow therapeutic index, because toxicity often limits the dose of 5-FU that can be administered, limiting its overall therapeutic usefulness.

Because of the association between the incorporation of 5-FU into RNA and dose-limiting toxicities, uridine has previously been examined for potential reduction of host toxicity. Uridine, a naturally occurring pyrimidine nucleoside, augments cellular UTP pools and competes with FUTP for incorporation into the RNA of hematopoietic progenitor and gastrointestinal mucosal cells, thereby attenuating 5-FU toxicity in these normal tissues

[4–8]. In mouse models, administration of uridine following 5-FU selectively reduces toxicity to normal tissues, permitting substantial 5-FU dose escalation and increasing the overall efficacy of 5-FU [4–8]. Preclinical and clinical studies have revealed that sustained uridine concentrations of at least 50 μmol/l are required to confer protection to normal tissues from the toxic effects of 5-FU [6]. Differences in uptake and utilization of uridine between tumor and normal tissues underlie uridine's ability to reduce the toxicities of 5-FU without proportionally reducing antitumor activity [3]. Both hematopoietic and gastrointestinal mucosal progenitors efficiently incorporate exogenous uridine (via the "salvage pathway"), whereas most other tissues, including malignant tumors, favor the de novo pathway of uridine nucleotide biosynthesis, in which free uridine is not an intermediate [3]. Thus, exogenous uridine is more effective in competing with FUTP for incorporation into RNA in normal tissues versus all solid tumors tested to date in murine systems. Although uridine has also been demonstrated to protect against 5-FU toxicity in humans, its low oral bioavailability and the requirement for central venous access for parenteral administration impair clinical utility [9–12].

PN401 (2′,3′,5′-tri-*O*-acetyluridine; Fig. 1; Wellstat Therapeutics Corporation, Gaithersburg, MD, USA), an orally active prodrug of uridine, represents a more effective uridine administration technique. PN401 is efficiently absorbed from the gastrointestinal tract and deacetylated by nonspecific esterases, yielding uridine and acetate. In contrast to oral uridine, PN401 is not a substrate for the catabolic enzyme uridine phosphorylase and does not require the pyrimidine transporter for absorption. Consequently, administration of PN401 results in substantially more bioavailable uridine than does oral administration of uridine itself. Using this PN401 rescue, it has been possible to increase the therapeutic index of 5-FU in BALB/c mice bearing advanced transplants of Colon 26 adenocarcinoma. Further, in the latter tumor system, it has been possible to increase the dose of 5-FU resulting in a significant increase in antitumor activity without increased toxicity.

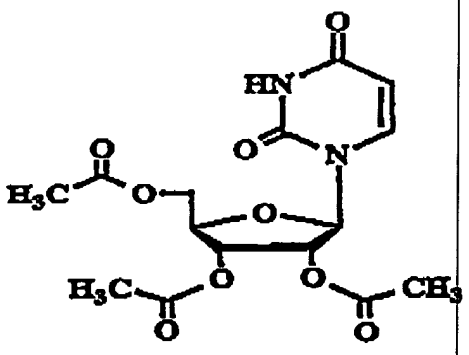

Fig. 1 Structure of PN401 showing 2′,3′,5′-tri-*O*-acetyluridine

Purpose

The primary objectives of the present study were to determine antitumor efficacy of 5-FU dose escalation enabled by delayed administration of PN401, to compare the efficacy of high-dose 5-FU plus PN401 with other 5-FU modulators or regimens and to evaluate the role of PN401 as an antidote to 5-FU overdose.

Experimental design

Murine tumor system

Frozen samples of Colon 26 adenocarcinoma were obtained from the National Cancer Institute (Frederick, MD, USA) and maintained by s.c. serial transplantation in BALB/c mice. For experimentation, 0.3 ml of a 5% tumor brei (in modified Dulbecco's phosphate-buffered saline at pH 7.2) prepared from 4–6 tumors was transplanted s.c. into 8-week-old BALB/c mice. Approximately 7–9 days later, when tumors were palpable, they were measured and the animals were distributed among the experimental groups of ten mice each so that animals carrying approximately equal-sized tumors were represented in each group.

Drug and chemicals

5-FU and leucovorin were obtained from the Sigma Chemical Co. (St Louis, MO, USA). Triacetyluridine and eniluracil were synthesized at Wellstat Therapeutics Corporation. 5-FU was dissolved in 0.85% NaCl solution immediately prior to use and administered by intraperitoneal (i.p.) injection such that the desired dose was contained in 0.1 ml/10 g of mouse weight. PN401 was suspended in 1% aqueous hydroxypropylmethylcellulose at a concentration of 80 mg/ml and administered by gavage.

Tumor measurements

Two axes of the tumor (the longest axis L and the shortest axis W) were measured with the aid of a vernier caliper. Tumor volume was estimated according to the formula:

$$\text{Tumor volume (mm}^3) = L \times W^2 \times \frac{\pi}{6}.$$

Statistical evaluation

Student's t test was used for the statistical evaluation of difference in mean tumor size between groups of treated

nice. Differences between groups with a statistical probability of 0.05 or less were considered significant.

Toxicity measurements

Animal body weights were recorded immediately before and weekly after the initiation of treatment. Weight change was calculated as a percentage of the initial body weight of the animals.

Groups of ten female BALB/c 8-week-old mice bearing subcutaneous Colon 26 adenocarcinoma were treated with weekly 3× i.p. injections of 5-FU alone at MTD (100 mg/kg) or at escalated doses of 5-FU (150, 175, and 200 mg/kg) with delayed administration of PN401. PN401 was administered as 2,000 mg/kg PO starting 2 h after 5-FU dose, q8h ×5. Tumor volumes were measured weekly, with final tumor volumes measured 1 week after the last 5-FU dose. Analysis of 5-FU pharmacokinetics was performed according to the methods described elsewhere [13].

Then a comparison was made to several 5-FU dosing schedules corresponding to common clinical regimens for 5-FU administration. 5-FU is often administered on a daily 5× schedule (repeated every 3–4 weeks) with or without leucovorin (LV). Eniluracil (EU) inhibits 5-FU degradation and thereby enables oral 5-FU administration and furthermore provides plasma 5-FU pharmacokinetics approximating prolonged intravenous infusion. In the following comparative regimens, 5-FU was administered at MTD for BALB/c mice: Regimen A: 5-FU 200 mg/kg i.p. days 1 and 7, PN401 2,000 mg/kg PO starting 2 h after 5-FU dose every 8 h for a total of five doses (following both on 2 and 7 days 5-FU); Regimen B: 5-FU 35 mg/kg i.p. for 5 days; Regimen C: 5-FU 35 mg/kg i.p. + LV 100 mg/kg i.p. for 5 days; Regimen D: 5-FU 2 mg/kg + PO EU 2 mg/kg PO days 1–9. Groups each comprised ten female BALB/c 8-week-old mice bearing subcutaneous Colon 26 adenocarcinoma. Tumor volume was measured on days 7 and 14.

Finally, to investigate the best timing of the first dose of PN401 relative to 5-FU administration as an important determinant of the effectiveness of PN401 in ameliorating the adverse effects of 5-FU, female BALB/c mice (nontumor bearing) received a lethal dose of 5-FU (400 mg/kg i.p.). Groups of mice then received oral PN401 beginning 2, 24, or 48 h after the 5-FU dose. Survival was monitored for 30 days.

Results

The pretreatment mean tumor volume was approximately 200 mm^3. In untreated animals, the tumors grew to a mean size of about 3,400 mm^3. 5-FU at its normal MTD (100 mg/kg) partially inhibited tumor growth, resulting in a final mean tumor volume of about 1,500 mm^3, and no regressions were observed. In the group that received 200 mg/kg/week 5-FU, a dose tolerated only due to toxicity reduction by PN401, durable complete tumor regressions were observed in 8/10 mice and partial regressions (> 50% regression) in the remaining two mice. At 175 mg/mg dose of 5-FU with PN401, a CR rate of 40% and a PR rate of 60% was obtained. 5-FU at 150 mg/kg/week + PN401 yielded 10% PR rate and mean tumor volume of 690 mm^3 (Table 1). Significantly, the improved antitumor response of high-dose 5-FU enabled by PN401 was achieved without a toxicity penalty as determined by body weight changes (Table 1). Analysis of 5-FU pharmacokinetics displayed nonlinearity as a function of administered dose in mice. In female CD-1 mice receiving 5-FU by i.p. injection the "area under the curve" (AUC) for plasma 5-FU at a dose of 100 mg/kg is 135 μmol h/ml (mean for five mice). At a dose of 150 mg/kg, the AUC is 197 μmol h/ml and at 200 mg/kg, the AUC is 485 μmol h/ml. Thus, the twofold increase in 5-FU MTD in mice receiving PN401 corresponds to about a fourfold increase in the plasma AUC of 5-FU.

In the comparison study, the tumor volumes on days 7 and 14 are indicated in Table. 2 and 3. 5-FU at 200 mg/kg with delayed oral PN401 induced significant tumor regression (9/10 CR). The other regimens (5-FU daily × 5 + LV and 5-FU PO daily × 9 + EU resulted in the inhibition of tumor growth, but no regressions were observed, even though toxic weight loss was greater in these groups than in mice treated with high-dose 5-FU plus PN401.

In the timing of administration study, after a single lethal dose of 5-FU (400 mg/kg i.p.), mice that did not receive PN401 died by day 12 while most animals in the other three groups were alive at day 31 (Fig. 2). Moreover, the proportion of mice surviving was highest in the group that received PN401 at 2 h followed by 24 and 48 h. A significant improvement in survival was seen even when PN401 was administered 48 h after 5-FU.

About 12 separate variations on the Colon 26 experiments (slightly different dosing regimens, different comparison arms) were done, all corroborating the reported results. Such variations included individual dose as well as the cumulative dose of PN401 administered following 5-FU, timing between 5-FU and the first dose of PN401 ranging from 2 to 24 h, and the number of doses of PN401 required following 5-FU.

Discussion

This study has shown that there is a threshold 5-FU dose after which the efficacy is dramatically improved—in mice, that threshold is a dose of > 150 mg/kg/week (i.e. 175 and 200 mg/kg/week), and the increased efficacy correlates with about a fourfold increase in the AUC of 5-FU. This finding is consistent with previous studies including the use of injected uridine [4], uridine

Table 1 Results of the study to determine MTD of 5-FU given alone versus with PN401 and responses (tumor volume)

Treatment (mg/kg)	Tumor vol. (mm^3)	Control (%)	No. dead/no. treated	Regressions		
				PR	CR	Total
Control (no 5-FU)	3,406 ± 263	–	3/10	0/10	0/10	0/10
5-FU 100	1,503 ± 267	44.1	0/10	0/10	0/10	0/10
5-FU 200 + PN401	12 ± 10	0.3	0/10	2/10	8/10	10/10

Tumor: subcutaneous Colon 26 adenocarcinoma, initial size 198 ± 14 mm^3; 5-FU: i.p. injection, weekly 3×; PN401: five oral doses at 8 h intervals, beginning 2 h after 5-FU; evaluation: 7 days after the last of three weekly 5-FU doses. The MTD of 5-FU alone was 100 mg/kg/week whereas the MTD of 5-FU when given with PN401 was 200 mg/kg/week. A complete response (CR) of 80% and partial response (PR) of 20% was observed with 5-FU (200 mg/kg) + PN401 compared to CR of 40% and PR of 60% with 5-FU (175 mg/kg) + PN401, no CR and PR of 10% with 5-FU (150 mg/kg) + PN401 while no responses were seen with 5-FU (100 mg/kg) + PN401 *PR* partial response, > 50% decrease in tumor size; *CR* complete response, no tumor detectable

Table 2 Results of comparative regimen study: evaluating responses of 5-FU given alone, with LV, EU, and PN401

Group	Day 7 tumor vol. (mm^3)	Day 14 tumor vol. (mm^3)
Control	1,191 ± 215	1,230 ± 154
5-FU 200 + PN401	13 ± 2	0.5 ± 0.4
5-FU 35×5 d	266 ± 71	633 ± 144
5-FU 35×5 d + LV	159 ± 51	515 ± 122
5-FU 2×9 d + EU	225 ± 47	924 ± 98

Initial tumor size 82 ± 9 mm^3. In the regimen comparison study, tumor volume measured on day 7 and day 14 showed that the best response was achieved with PN401 when compared to EU and LV
LV leucovorin, *EU* ethynyluracil (DPD inhibitor that permits oral 5-FU administration)

diphosphoglucose (UDPG) [14] and PN401 in combination with 5-(benzyloxybenzyl)barbituric acid acyclonucleoside (BBBA), an inhibitor of uridine phosphorylase (UrdPase) [15], indicating that these agents can allow the escalation of 5-FU doses for better chemotherapeutic efficacy. This is the first report that delayed administration of PN401 alone is sufficient for enabling dose escalation of 5-FU to a degree sufficient to induce regressions of the Colon 26 adenocarcinoma.

5-FU is administered in a broad range of dosing regimens and no single dose schedule has ever emerged as clearly superior at maximum tolerated doses [1–3], and the trend towards superiority of one regimen over another may depend on the tumor type. At standard doses, inhibition of thymidylate synthase (TS) apparently predominates as a mechanism of antitumor cytotoxicity, and this inhibition is perhaps best exploited by continuous infusion of 5-FU, although resistance through compensatory overexpression of TS almost inevitably occurs. Acute TS inhibition is maximal at relatively low doses of 5-FU; the problem is to maintain durable inhibition. Unlike TS inhibition, RNA-directed cytotoxicity of 5-FU cannot be saturated at clinically achievable 5-FU concentrations, presenting, in principle, an opportunity for increased activity with dose escalation. However, this mechanism is operative only when concentrations of the 5-FU anabolite FUTP are sufficient to compete with endogenous UTP for incorpora-

Table 3 Comparative 5-FU pharmacokinetics after various clinical dosing regimens

5-FU regimen	5-FU dose (mg/m^2/day)	Dose rate	No. of doses/month	Total dose (mg/m^2/month)	Cl (l/h/m^2)	$AUC_{0-24\ h}$ (μmol h)	$AUC_{0-30\ days}$ (μmol h)
Daily × 5	375	1–2 min	5	1,875	42.2	73.5	368
Weekly bolus	500	20 min	4	2,000	65.3	55.9	224
Weekly bolus	500	2 min	4	2,000	42.5	103	412
PN401 weekly bolus	1,400	30 min	3	4,200	24.1	466	1,398
Continuous infusion	146	Continuous	30	4,380	183	7.2	216
120 h infusion	1,000	120 h	5	5,000	156	50.4	252
Weekly 24 h infusion	1,500	24 h	4	6,000	127	96	384
72 h infusion	2,000	72 h	3	6,000	159	156	468

The comparison of Phase I studies [13, 20] studies and various other intravenous schedules of 5-FU [3] have showed disproportionate increases in AUC and corresponding decreases in clearance with increasing 5-FU dose (range 1,200–1,950 mg/m^2). While the dose increases over a standard dose ranged from 2.4 to 3.9-fold, average 5-FU exposures, as measured by AUC, ranged from 5.6 to 18.6-fold [21, 22]. The intrapatient variability in exposure decreased with increased 5-FU dose. With high-dose 5-FU enabled by PN401, clearance is reduced to approximately threefold than that observed with the maximum inhibition of DPD with EU [23]

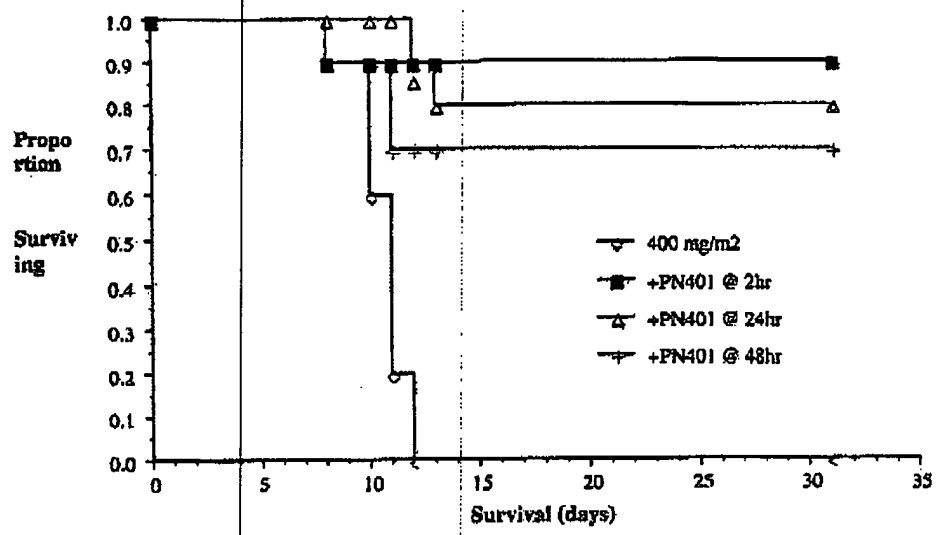

Fig. 2 Survival in mice treated with 400 mg/m^2 5-FU and PN401 and relation to time of dosing. Mice that did not receive PN401 died by day 12, while the other three groups were alive at day 31. Moreover, the proportion of mice surviving was highest in the group which received PN401 at 2 h followed by 24 and 48 h

tion into nuclear RNA, which requires high extracellular concentrations of 5-FU. Thus, bolus administration of 5-FU may be necessary for exploitation of RNA-directed 5-FU toxicity, but dose-limiting 5-FU toxicities intervene. The fact that uridine is an effective antidote for both gastrointestinal and hematologic toxicities due to bolus 5-FU implies that RNA-directed effects account for dose-limiting toxicities in this situation, since delayed uridine does not reverse inhibition of TS by FdUMP [16].

RNA-related toxicities of 5-FU are largely a function of the concentration × time product, equivalent to the AUC for plasma pharmacokinetics [17]. It is noteworthy that although different total amounts of 5-FU are administered in various clinical regimens, the AUC for intact 5-FU (versus total 5-FU plus inactive 5-FU catabolites) during the course of periods of therapy of equivalent duration (i.e., 30 days, to account for different dosing schedules), is quite similar, whether 5-FU is administered by weekly bolus, daily × 5 bolus, 24 or 48 h infusion, or continuous 28 day infusion [3]. This suggests that 5-FU therapy is limited by an "AUC ceiling", integrated over a cycle of treatment. An important feature of 5-FU pharmacokinetics is that the clearance rate varies inversely with plasma concentration, due to saturable degradation processes, e.g., by the enzyme dihydropyrimidine dehydrogenase (DPD) [18]. A large fraction of an administered dose of 5-FU is degraded rather than anabolized to cytotoxic fluoropyrimidine nucleotides, especially during prolonged infusion, so that the AUC achieved is perhaps a more meaningful measure of 5-FU exposure than the administered dose. Thus, despite different total doses of 5-FU administered in various regimens, the systemic exposure to intact 5-FU per treatment cycle of equivalent duration is similar, and this may account for the therapeutic equivalence of the various regimens. However, it is tantalizing that the dose response for clinical activity of bolus 5-FU has a very steep slope [19], i.e., a relatively small increase in tumor exposure to intact 5-FU could in principle lead to a substantial increase in clinical efficacy.

Delayed uridine administration after high-dose 5-FU has been demonstrated to improve antitumor efficacy versus 5-FU at MTD in a variety of tumor models, and clinical implementation of this strategy has been attempted. Uridine itself is poorly absorbed, resulting in osmotic diarrhea at doses required to elevate plasma uridine into the therapeutic range, and intravenous administration of uridine is cumbersome due to the large amounts of uridine required, resulting in hyperthermia or phlebitis if the infusion rate and route are not carefully controlled [9–12]. In contrast, PN401 (triacetyluridine) acts as a lipophilic prodrug of uridine that is efficiently absorbed and rapidly deacetylated, yielding more bioavailable circulating uridine than the oral administration of uridine itself. The strong antitumor efficacy of high-dose 5-FU with delayed PN401, with less toxicity (weight loss) than was observed with lower doses of 5-FU without PN401, which only attenuated tumor growth without inducing regressions, indicates that PN401 rescue was relatively selective for normal tissues versus the tumors.

The clinical feasibility of 5-FU dose escalation with PN401 has been demonstrated in phase I studies [13, 20]. These studies have showed disproportionate increases in AUC and corresponding decreases in clearance with increasing 5-FU dose (range 1,200–1,950 mg/m^2) (Table 3; Fig. 3). While the dose increases over a standard dose ranged from 2.4 to 3.9-fold, average 5-FU exposures, as measured by AUC, ranged from 5.6 to 18.6-fold [21, 22]. The intrapatient variability in exposure decreased with increased 5-FU dose. With high-dose 5-FU enabled by PN401, clearance is reduced to approximately threefold than that observed with the maximum inhibition of DPD with EU [23]. In an ongoing phase III clinical study of high-dose 5-FU in

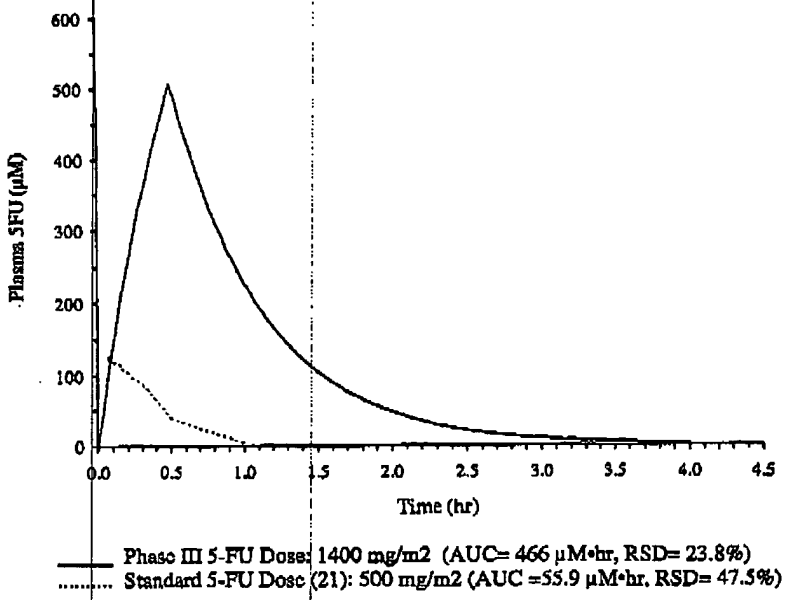

Fig. 3 Plasma 5-FU concentration–time profile: phase III dose versus conventional dose. Comparison of AUC achieved with high-dose 5-FU enabled by delayed oral administration of PN401 versus standard 5-FU bolus dose showed disproportionate increases in AUC when 5-FU was given with PN401

pancreatic cancer, patients receive 1,400 mg/m^2 5-FU per week 3× with 1 week rest, with PN401 6 g administered q8h for eight doses, beginning 8 h after a rapid (30 min) 5-FU infusion. In a phase II clinical study in gastric cancer conducted by SWOG, patients received 5-FU at 1,200 mg/m^2 per week 6× with leucovorin, followed 8 h after 5-FU with PN401 6 g q8h for eight doses. High-dose 5-FU enabled with PN401 is also in principle compatible with combination chemotherapy involving the same spectrum of agents used with standard doses of 5-FU, e.g., oxaliplatin, irinotecan, cisplatin, epirubicin, taxotere.

These experiments also indicated that the timing of the first dose of PN401 may be an important determinant of 5-FU toxicity reduction and the degree of safe 5-FU dose escalation as mice can tolerate higher 5-FU doses if PN401 rescue starts earlier than later. PN401 used to rescue mice from the lethal toxicity of 5-FU not only allows administration of high 5-FU doses to tumors unresponsive to conventional doses of 5-FU but also entails that PN401 can be used as a *antidote* even when used after 48 h in patients who have received an accidental overdose or severe untoward toxicity of 5-FU, in particular RNA-medicated host toxic effects. This issue is particularly important in situations including pump malfunction or ingestion of excessive quantities of 5-FU prodrugs like capecitabine. Moreover, the potential of PN401 in DPD-deficient patients to allow 5-FU treatment also warrants investigation, in particular when they have 5-FU sensitive tumors, which may affect up to about 3% of the population [24].

In summary, the results of this study demonstrate that uridine exposure resulting from PN401 not only allows administration of high 5-FU doses to tumors unresponsive to conventional doses of 5-FU but also entails that PN401 can be used as an antidote even when used up to 48 h after a 5-FU overdose. Because of the nonlinearity of 5-FU pharmacokinetics, the two times increase in the MTD of 5-FU was associated with a fourfold increase in 5-FU exposure (i.e., AUC). Future exploratory studies aiming at evaluating the role of PN401 in DPD-deficient patients as well as clinical trials to further confirm this biochemical modulation with PN401 is warranted.

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Use of Uridine Rescue to Enhance the Antitumor Selectivity of 5-Fluorouracil[1]

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ABSTRACT

We examined the ability of uridine to increase the therapeutic index of 5-fluorouracil (FUra) against C57BL/6 × DBA/2 F_1 mice bearing a Day 1 B16 melanoma or L1210 leukemia. FUra (400, 600, or 800 mg/kg, i.p.) followed in 24 hr by a 5-day s.c. infusion with uridine (5 g/kg/day, s.c.) was compared with the maximum tolerated dose of FUra (200 mg/kg, i.p.) plus a 5-day infusion with 0.9% NaCl solution. High-dose FUra plus delayed infusion with uridine was more effective than FUra (200 mg/kg) in inhibiting the growth of the B16 melanoma. High-dose FUra plus uridine rescue was, however, no more effective than FUra (200 mg/kg) in increasing the survival times of mice bearing the L1210 leukemia.

To see if uridine rescue from FUra toxicity correlated with effects against a sensitive normal tissue, bone marrow nucleated cellularity of normal, non-tumor-bearing mice was monitored after drug treatment. In mice treated with FUra (200 mg/kg) followed in 24 hr by a 5-day infusion with either uridine (5 g/kg/day) or 0.9% NaCl solution, there was not as great a decrease in cellularity at the nadir with uridine, and, in addition, uridine accelerated recovery as compared to 0.9% NaCl solution. Furthermore, uridine (5 g/kg/day), but not thymidine (dThd) (5 g/kg/day) or 2′-deoxyuridine (dUrd) (5 g/kg/day), had a sparing effect on the depression in bone marrow nucleated cellularity seen at the nadir on Day 4 after Fura (200 mg/kg).

The specificity of uridine to rescue mice from the lethal toxicity of the related fluorinated pyrimidines, 5-fluorouridine and 5-fluoro-2′-deoxyuridine, was also examined. Mice were treated with 5-fluorouridine (250 mg/kg, i.p.) followed in 24 hr by a 5-day infusion with uridine (1, 5, or 10 g/kg/day), dThd (1, 5, or 10 g/kg/day), or dUrd (1 or 5 g/kg/day). Uridine (1, 5, or 10 g/kg/day) rescued mice from the lethal toxicity of 5-fluorouridine, whereas dThd or dUrd was ineffective. Similarly, a 5-day infusion with uridine, but not dThd or dUrd, rescued mice from the lethal toxicity of 5-fluoro-2′-deoxyuridine (1800 mg/kg, i.p.).

INTRODUCTION

Major disruptions in DNA and RNA synthesis following treatment with FUra[3] are due to the anabolism of the drug to FdUMP and FUTP (10). In different tumor cell lines, either the DNA- or RNA-directed actions of FUra may be the principal determinants of its cytotoxicity (5, 21, 25). Furthermore, there is a specificity for dThd, uridine, or dUrd for reversing the cytotoxicity of FUra (5, 23, 26). As for normal host tissues, the importance of the RNA-directed actions of FUra, FUrd, or FdUrd to their dose-limiting gastrointestinal toxicity in CBA/J mice correlates best with the incorporation of FUTP, derived from these fluorinated pyrimidines, into intestinal RNA rather than with levels of FdUMP (11).

Our observation that delayed administration of a continuous s.c. infusion of uridine, but not dThd or dUrd, rescues mice from the lethal toxicity of FUra is consistent with FUra/RNA and not inhibition of thymidylate synthetase as a determinant of the toxicity of FUra (14, 15). It seemed possible, therefore, that if differences existed in the RNA- and DNA-directed actions of FUra in tumor as compared to host normal tissues, a selective rescue of normal tissues with uridine could then increase the therapeutic index of FUra.

In this report, we describe our studies with transplantable mouse tumors which indicate the potential of high-dose FUra plus uridine rescue to increase the therapeutic index of FUra. Furthermore, we present evidence that FUra-induced toxicity to a sensitive host tissue can be selectively reversed with uridine but not with either dThd or dUrd. Finally, we have demonstrated that uridine, but not dThd or dUrd, also rescues normal, non-tumor-bearing mice from the lethal toxicity of FUrd or FdUrd.

MATERIALS AND METHODS

Drugs. FUra and dThd were obtained from Dr. V. L. Narayanan, Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. Uridine and dUrd were obtained from Sigma Chemical Co. (St. Louis, Mo.). FUrd and FdUrd were obtained as a gift from Hoffmann-La Roche Inc. (Nutley, N. J.). Sodium pentobarbital was obtained from Veterinary Laboratories, Inc. (Lenaxa, Kans.).

FUra was dissolved in 2% $NaHCO_3$ (w/v) just prior to use. FUrd or FdUrd was dissolved in 0.9% NaCl solution (w/v) just prior to use. All drugs were administered i.p. in volumes of 0.01 ml/g of mouse body weight. Uridine, dThd, and dUrd were each prepared in 0.9% NaCl solution, and all solutions were sterilized by passage through 0.22-μm Millipore filters just prior to administration by continuous s.c. infusion. In all experiments, mice which did not receive drug(s) were given an equivalent volume of 0.9% NaCl solution.

Animals and Tumors. Male C57BL/6 × DBA/2 F_1 (hereafter called B6D2F_1) mice, weighing 20 to 23 g, were obtained from Harlan-Sprague-Dawley Laboratories (Madison, Wis.). Male C57BL/6 mice, weighing 20 to 23 g, were obtained from Microbiological Associates (Bethesda, Md.). Male DBA/2 mice, weighing 18 to 23 g, were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, Mass.). Animals were caged in an air-conditioned room lighted from 8 a.m. to 8 p.m. and had free access to standard Purina laboratory chow diet and tap water. The L1210 lymphocytic leukemia in the ascites form was passaged weekly in DBA/2 mice. The solid B16 melanoma was passaged every 10 to 14 days in C57BL/6 mice by s.c. implantation of 0.25 ml of a 1/5 (w/v) tumor brei. The tumors were obtained from E. G. and G. Mason Research Institute (Worcester, Mass.).

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[3] The abbreviations used are: FUra, 5-fluorouracil; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; FUTP, 5-fluorouridine 5′-triphosphate; dThd, thymidine; dUrd, 2′-deoxyuridine; FdUrd, 5-fluoro-2′-deoxyuridine; FUrd, 5-fluorouridine; ILS, increase in median life span.

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Continuous s.c. Infusion. Mice were anesthetized with a single s.c. injection of sodium pentobarbital (75 mg/kg) prior to insertion of a s.c. cannula. The technique for continuous s.c. infusion with uridine, dThd, or dUrd has been described (13, 15).

Antitumor Activity of FUra plus Uridine Rescue. All chemotherapy experiments were routinely begun between 8 and 9 a.m. For experiments with the solid B16 melanoma, $B6D2F_1$ mice were anesthetized on Day 0 with a single s.c. injection of sodium pentobarbital (75 mg/kg), and 0.25 ml of a 1/5 (w/v) tumor brei was injected into the axillary region with puncture in the inguinal region. On Day 1, the mice were randomly distributed into groups of 8 each, and they were then each given a single i.p. injection of either 0.9% NaCl solution or FUra (200, 400, 600, or 800 mg/kg). Twenty-four hr later, a s.c. infusion with either 0.9% NaCl solution or uridine (5 g/kg/day) was begun and continued for 5 days. On Day 16 after tumor inoculation, the mice were sacrificed by cervical dislocation. The tumors were excised surgically, and the weight of each tumor was determined. For experiments with the L1210 tumor, $B6D2F_1$ mice were given a single i.p. injection of 1×10^5 L1210 ascites cells on Day 0. On Day 1, the mice were randomly distributed into groups of 6 each, and they were then given a single i.p. injection of either 0.9% NaCl solution or FUra (200, 400, 600, or 800 mg/kg). Twenty-four hr later, a s.c. infusion with either 0.9% NaCl solution or uridine (5 g/kg/day) was begun and continued for 5 days. Animals were observed for individual days of death, and the median survival time and percentage of ILS were determined as described previously (8).

Effect of Chemotherapy on Bone Marrow Nucleated Cellularity. Normal, non-tumor-bearing $B6D2F_1$ mice were given a single i.p. injection of FUra (200 mg/kg) on Day 0. Twenty-four hr later, the mice were randomly distributed into groups of 20 each, and a s.c. infusion with either 0.9% NaCl solution or uridine (5 g/kg/day) was begun and continued for 5 days. Mice in groups of 4 each per treatment were sacrificed by cervical dislocation on Days 1, 3, 4, 6, and 8 after FUra, and the number of nucleated cells per femur was determined as described (7). In another experiment, normal, non-tumor-bearing $B6D2F_1$ mice were given a single i.p. injection of FUra (200 mg/kg) on Day 0. On Day 1, the mice were randomly distributed into groups of 4 each, and a s.c. infusion with 0.9% NaCl solution, uridine (5 g/kg/day), dThd (5 g/kg/day), or uridine (5 g/kg/day) was begun. Seventy-two hr after the infusion was begun, the animals were sacrificed by cervical dislocation, and the number of nucleated cells per femur was determined. Preliminary experiments showed that the nadir in bone marrow nucleated cellularity occurred on Day 4 after FUra (200 mg/kg) treatment.

Effect of Either Uridine, dThd, or dUrd Infusion to Rescue Mice from the Lethal Toxicity of FUrd or FdUrd. In preliminary experiments with normal, non-tumor-bearing $B6D2F_1$ mice, we determined that the approximate 90% lethal dose for a single i.p. dose of either FUrd or FdUrd was 250 and 1800 mg/kg, respectively. For experiments with FUrd, mice were given a single i.p. injection of FUrd (250 mg/kg). Twenty-four hr later, the mice were randomly distributed into groups of 6 each, and a s.c. infusion of either 0.9% NaCl solution or uridine (1, 5, or 10 g/kg/day) was immediately begun and continued for 5 days. Survivors were determined daily for 30 days after FUrd. Similar experiments were also done in which mice received a single i.p. injection of FUrd (250 mg/kg) followed in 24 hr by a 5-day infusion with 0.9% NaCl solution, dThd (1, 5, or 10 g/kg/day), or dUrd (1 or 5 g/kg/day). Experiments similar to those with FUrd were also done in which mice received a single i.p. injection of FdUrd (1800 mg/kg) followed in 24 hr by a 5-day infusion with 0.9% NaCl solution, uridine (1, 5, or 10 g/kg/day), dThd (1, 5, or 10 g/kg/day), or dUrd (1 or 5 g/kg/day). The dosage schedules of uridine, dThd, or dUrd which were used did not cause any marked gross toxicity (15).

RESULTS

Effect of High-Dose FUra plus Uridine Rescue on the Growth of Mouse B16 Melanoma. In previous studies from this laboratory, we have found that FUra at 200 mg/kg was the maximum tolerated dose when given as a single i.p. injection against either normal or L1210 tumor-bearing $B6D2F_1$ mice (6, 15, 16). In order to confirm that FUra (200 mg/kg) was also the maximum tolerated dose against mice bearing a Day 1 B16 melanoma implanted s.c., we compared the effect of either 0.9% NaCl solution or FUra (200, 400, or 600 mg/kg) plus 0.9% NaCl solution infusion on tumor growth and survival. Table 1 (Experiment 1) shows the results obtained when tumor weights were determined from control and FUra-treated mice on Day 16 after tumor implantation. As can be seen, FUra (400 or 600 mg/kg) plus 0.9% NaCl solution infusion inhibited tumor growth as compared to FUra (200 mg/kg) plus 0.9% NaCl solution infusion, the latter of which was ineffective as compared to control. However, FUra (200 mg/kg) plus 0.9% NaCl solution infusion resulted in 8 of 8 Day 16 survivors, whereas FUra (400 or 600 mg/kg) plus 0.9% NaCl solution infusion resulted in 5 of 8 and 3 of 8 Day 16 survivors, respectively. These results, which are consistent with our previous studies on FUra toxicity, indicate that FUra (200 mg/kg) was the maximum tolerated dose tested against the mouse B16 melanoma. In addition, a 5-day infusion with 0.9% NaCl solution did not appear to alter FUra toxicity.

To determine if uridine rescue could increase the therapeutic index of FUra, we compared the effect of high-dose FUra plus uridine rescue with the maximum tolerated dose of FUra alone on the growth of a Day 1 B16 melanoma, implanted s.c. Table 1 shows the results obtained when tumor weights were determined from control and drug-treated mice on Day 16 after tumor implantation. As can be seen (Experiments 2 and 3), FUra (400 or 600 mg/kg) plus uridine rescue were both more effective than was FUra (200 mg/kg) alone in inhibiting tumor growth. Similarly in Experiment 4, FUra (600 or 800 mg/kg) plus uridine rescue were both more effective than was FUra (200 mg/kg) alone in inhibiting tumor growth. The fact that all animals treated with the higher doses of FUra remained alive at Day 16 indicated that uridine had prevented the anticipated host toxicity. These results with the mouse B16 melanoma indicated that a single i.p. injection of high-dose FUra followed in 24 hr by a 5-day infusion with uridine was more tumor inhibitory than the maximum tolerated dose of FUra alone.

Effect of High-Dose FUra plus Uridine Rescue on the Survival of Mice Bearing the Ascitic L1210 Leukemia. To determine if uridine rescue could increase the therapeutic index of FUra against a mouse leukemia, we compared the effect of high-dose FUra plus uridine rescue with the optimal dose of FUra alone on the survival times of mice bearing a Day-1 L1210 leukemia, implanted i.p. As shown in Table 2 (Experiment 1), FUra (400 mg/kg) plus uridine rescue (5 g/kg/day) was no more effective than was FUra (200 mg/kg) alone in that the ILS was 63 and 69%, respectively. Similarly in Experiments 2 and 3, FUra (600 or 800 mg/kg) resulted in an ILS which was not greater than that seen with FUra (200 mg/kg) alone. These results with the mouse L1210 leukemia indicated that a single i.p. injection of high-dose FUra followed in 24 hr by a 5-day infusion with uridine did not produce an ILS greater than that which occurred with the optimal dose of FUra alone.

Effect of Uridine, dThd, or dUrd Infusion on the FUra-induced Depression of Bone Marrow Nucleated Cellularity. To see if uridine rescue from the toxicity of a FUra correlated with effects against a sensitive normal tissue in mice, we monitored bone marrow nucleated cellularity after drug treatment. As can be seen (Chart 1), in mice treated with a single i.p. injection

Table 1

Effect of FUra plus uridine rescue on the growth of B16 melanoma in mice

Experiment	Treatment: Day 1, FUra (mg/kg)	Treatment: Days 2–6, 0.9% NaCl solution infusion	Treatment: Days 2–6, Uridine infusion (5 g/kg/day)	Individual days of death	Day-16 survivors/total treated	Tumor wt (mg)	Tumor wt (% of control)[a]
1		+	−		8/8	2530 ± 308[b]	
	200	+	−		8/8	2284 ± 343	90
	400	+	−	13, 14, 14	5/8	1142 ± 100[c]	45
	600	+	−	11, 11, 11, 12, 13	3/8	910 ± 139[c]	36
2		−	+		8/8	1137 ± 225	
	200	+	−		8/8	1229 ± 114	108
	400	−	+		8/8	801 ± 134[c]	70
	600	−	+		8/8	497 ± 109[d]	43
3		−	+		8/8	2828 ± 288	
	200	+	−		8/8	2802 ± 571	99
	400	−	+		8/8	1073 ± 221[c]	38
	600	−	+		8/8	968 ± 114[d]	34
4		−	+		8/8	1823 ± 435	
	200	+	−		8/8	1554 ± 284	85
	400	−	+		8/8	545 ± 96[d]	30
	600	−	+		8/8	267 ± 81[f]	15

[a] Groups of 8 mice were inoculated with 0.25 ml of a 1/5 (w/v) B16 melanoma brei by s.c. injection on Day 0. On Day 1, mice received a single i.p. injection of FUra, as indicated. Twenty-four hr later, a s.c. infusion of either 0.9% NaCl solution or uridine (5 g/kg/day), as indicated, was begun and continued for 5 days. Survivors were checked daily. Survivors were sacrificed on Day 16 after tumor implantation, and the tumors were dissected free and weighed.

[b] Mean ± S.E. of 8 mice per treatment group on Day 16.

[c] $p < 0.05$ as compared to FUra (200 mg/kg) by Student's t test analysis.

[d] $p < 0.005$ as compared to FUra (200 mg/kg) by Student's t test.

[e] $p < 0.01$ as compared to FUra (200 mg/kg) by Student's t test.

[f] $p < 0.001$ as compared to FUra (200 mg/kg) by Student's t test.

Table 2

Effect of FUra plus uridine rescue on the survival times of mice bearing L1210 leukemia

Experiment	Treatment: Day 1, FUra (mg/kg)	Treatment: Days 2–6, uridine (g/kg/day)	Median survival time (days)	Range of individual survival times (days)	Increase in median survival time[a] (%)
1		5	8.0	8–13	
	200		13.5	7–15	69
	400	5	13.0	12–16	63
2		5	9.0	9–10	
	200		14.0	9–15	47
	600	5	13.0	9–21	39
3		5	9.0	8–10	
	200		14.0	14–15	56
	600	5	13.0	8–16	45

[a] Groups of 6 mice each were inoculated with 1×10^5 L1210 cells by i.p. injection on Day 0. On Day 1, mice received a single i.p. injection of FUra, as indicated, followed in 24 hr by a s.c. infusion of uridine for 5 days. Animals not receiving drug were given an equivalent volume of 0.9% NaCl solution. Animals were monitored for day of death, and median survival times were determined.

of FUra (200 mg/kg) followed in 24 hr by a 5-day infusion with uridine (5 g/kg/day), there was not as great a decrease, as compared to 0.9% NaCl solution infusion, in the total number of bone marrow nucleated cells per femur on Days 3, 4, 6, and 8 after FUra. In addition, by Day 8, bone marrow nucleated cellularity had almost recovered in mice given a uridine infusion in contrast to a 0.9% NaCl solution infusion (82 and 49% of Day 0 control, respectively).

To determine the nucleoside specificity of this effect, we measured bone marrow nucleated cellularity at the nadir (Day 4) after FUra in mice treated with a single i.p. injection of FUra (200 mg/kg) followed in 24 hr by an infusion with either uridine (5 g/kg/day), dThd (5 g/kg/day), or dUrd (5 g/kg/day). As shown in Table 3, only uridine had a sparing effect on bone marrow nucleated cell numbers compared to 0.9% NaCl solution (25.0 and 13.6% of Day 0 control, respectively). In contrast, neither dThd nor dUrd prevented the FUra-induced depression in bone marrow cell numbers (13.2 and 16.5% of control, respectively).

Effect of Uridine, dThd, or dUrd Infusion to Rescue Mice from the Lethal Toxicity of FUrd or FdUrd. As is the case with FUra, the dose-limiting toxicity in mice of FUrd or FdUrd is more closely related to FUra/RNA formation than with FdUMP levels (11). We hypothesized, therefore, that uridine which rescues mice from the lethal toxicity of FUra (14, 15) would also be an effective rescue agent for FUrd or FdUrd.

Treatment of mice with a single i.p. injection of FUrd (250 mg/kg) followed in 24 hr by a 5-day infusion with uridine (1, 5, or 10 g/kg/day) rescued mice from the lethal toxicity of FUrd (Table 4,

Experiment 1). In contrast, dThd (1, 5, or 10 g/kg/day) or dUrd (1 or 5 g/kg/day) both failed to rescue mice from the lethal toxicity of a single i.p. injection of FUrd (250 mg/kg) (Table 4, Experiments 2 and 3, respectively). In the next set of experiments, mice were treated with a single i.p. injection of FdUrd (1800 mg/kg) followed in 24 hr by a 5-day infusion with either uridine, dThd, or dUrd. As can be seen (Table 5, Experiment 1), uridine (1, 5 or 10 g/kg/day) rescued mice from the lethal toxicity of FdUrd. In contrast, neither dThd (1, 5, or 10 g/kg/day) nor dUrd (1 or 5 g/kg/day) (Table 5, Experiments 2 and 3, respectively) rescued mice from the lethal toxicity of FdUrd.

DISCUSSION

In a previous report from this laboratory, we demonstrated that mice can be rescued from the lethal toxicity of a single i.p. injection of FUra with a uridine infusion begun 24 hr after FUra and continued for 5 days. In contrast, infusions with either dThd or dUrd failed to rescue mice from the lethal toxicity of FUra (14, 15). These experiments led us to examine whether delayed infusion with uridine could increase the therapeutic efficacy of FUra against tumor-bearing mice. The results reported herein demonstrate that uridine rescue increased the therapeutic index of FUra against the mouse B16 melanoma. The use of uridine rescue allowed a potentially lethal dose of FUra to be administered, and while host toxicity was substantially reduced, the antitumor action of FUra was retained. The therapeutic superiority of high-dose FUra plus uridine rescue was related to the fact that the maximum tolerated dose of FUra, when given alone, failed to inhibit the growth of the B16 melanoma, whereas high-dose FUra plus uridine rescue resulted in significant inhibition of tumor growth. The biochemical and/or pharmacological basis by

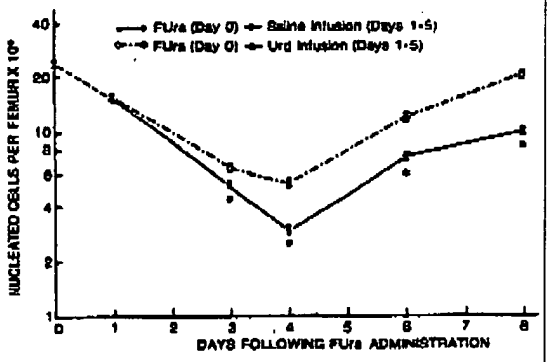

Chart 1. Serial changes in the numbers of nucleated cells per femur of mice following chemotherapy. Mice were each treated with a single i.p. injection of FUra (200 mg/kg). Twenty-four hr later, a s.c. infusion of uridine (5 g/kg/day) (○) or 0.9% NaCl solution (●) was begun and continued for 5 days. Mice in groups of 4 each per treatment were sacrificed on the days indicated, and the number of nucleated cells per femur was determined. Measurements were also made of untreated Time 0 control (×) and 24 hr after FUra (200 mg/kg) (×) groups. The values obtained of nucleated cells for the paired femora were averaged, and the results were expressed as the mean of 4 mice per treatment group. *Bars*, S.E. *, $p < 0.05$ compared to uridine (*Urd*) infusion by Student's *t* test.

Table 3

Effect of infusion with uridine, dThd, or dUrd on the FUra-induced depression of the numbers of nucleated cells per femur of mice

Group	Treatment: Day 0, FUra (mg/kg)	Days 1–4: Drug	Days 1–4: g/kg/day	Nucleated cells/femur × 10^{-6}[a]	% of control
1				21.8 ± 0.32[b]	
2	200			2.97 ± 0.10	13.6
3	200	Uridine	5	5.45 ± 0.13[c,d]	25.0
4	200	dThd	5	2.88 ± 0.09	13.2
5	200	dUrd	5	3.58 ± 0.11[e]	16.5

[a] Groups of 4 mice each were given a single i.p. injection of FUra followed in 24 hr by infusion with either 0.9% NaCl solution, uridine, dThd, or dUrd, as indicated, for 3 days. Mice were sacrificed, and the number of nucleated cells/femur was determined. The values obtained for the paired femora of each mouse were averaged and compared to an untreated Day 0 control group.

[b] Mean ± S.E. of 4 mice/treatment group.

[c] $p < 0.001$ compared to FUra (200 mg/kg) plus 0.9% NaCl solution infusion by Student's *t* test.

[d] $p < 0.0001$ compared to FUra (200 mg/kg) plus dUrd infusion by Student's *t* test.

[e] $p < 0.01$ compared to FUra (200 mg/kg) plus 0.9% NaCl solution infusion by Student's *t* test.

Table 4

Effect of infusion with uridine, dThd, or dUrd to rescue mice from the lethal toxicity of FUrd

Experiment	Treatment: Day 0, drug (mg/kg)	Treatment: Days 1–5, drug (g/kg/day)	Individual days of death	Day-30 survivors/total treated[a]
1	*FUrd*	*Uridine*		
	250		8, 8, 9, 10, 13	1/6
	250	1	8, 9, 17	3/6
	250	5	6, 10	4/6
	250	10	10	5/6
2	*FUrd*	*dThd*		
	250		8, 9, 9, 10, 11, 11	0/6
	250	1	8, 9, 9, 10, 13, 14	0/6
	250	5	6, 6, 9, 10, 12, 15	0/6
	250	10	6, 6, 6, 6, 7, 8	0/6
3	*FUrd*	*dUrd*		
	250		6, 6, 7, 7, 8, 8	0/6
	250	1	6, 7, 7, 8, 9, 9,	0/6
	250	5	6, 7, 8, 9, 13	1/6

[a] Groups of 6 mice each were given a single i.p. injection of FUrd followed in 24 hr by infusion with 0.9% NaCl solution, uridine, dThd, or dUrd as indicated for 5 days. Survivors were monitored daily for 30 days after FUrd treatment.

Table 5

Effect of infusion with uridine, dThd, or dUrd to rescue mice from the lethal toxicity of FdUrd

Experiment	Treatment: Day 0, drug (mg/kg)	Treatment: Days 1–5, drug (g/kg/day)	Individual days of death	Day 30-survivors/total treated[a]
1	*FdUrd*	*Uridine*		
	1800		6, 7, 7, 10	2/6
	1800	1		6/6
	1800	5	6	5/6
	1800	10	6	5/6
2	*FdUrd*	*dThd*		
	1800		6, 7, 8, 8, 10, 11	0/6
	1800	1	7, 8, 8, 11, 11	1/6
	1800	5	7, 8, 8, 8, 12	1/6
	1800	10	8, 8, 8, 10, 11	1/6
3	*FdUrd*	*dUrd*		
	1800		7, 7, 8, 8, 9, 10	0/6
	1800	1	7, 8, 8, 9, 10	1/6
	1800	5	7, 7, 8, 10, 12	1/6

[a] Groups of 6 mice each were given single i.p. injections of FdUrd followed in 24 hr by infusion with either 0.9% NaCl solution, uridine, dThd, or dUrd, as indicated, for 5 days. Survivors were monitored for 30 days after FdUrd treatment.

which uridine allows for selective rescue of host, normal tissues from FUra with retention of antitumor activity is, however, not clear.

In contrast to the increased therapeutic effect which we achieved with high-dose FUra plus uridine rescue against the B16 melanoma, the same dosage schedules afforded no advantage against the mouse L1210 leukemia. Although the L1210 leukemia responded to high-dose FUra plus uridine rescue, the percentage of ILS was not greater than that which was obtained with the optimal dose of FUra alone. It is not apparent, at this time, as to why high-dose FUra plus uridine rescue does not result in a therapeutic advantage against the mouse L1210 leukemia.

There is considerable evidence that the cytotoxicity of FUra is related to FUra/RNA formation (19). For example, the responsiveness of several animal and human tumors to FUra is related to its incorporation into RNA (4, 12, 17). It is of particular interest that Martin *et al.* (20) reported recently that treatment of BALB/c × DBA/2 F_1 mice bearing the advanced solid colon tumor 26 with FUra plus delayed administration of uridine given (by repeated injection) allows the maximum tolerated dose of FUra to be doubled without increasing host toxicity, thereby resulting in improved antitumor activity. In addition, their biochemical studies indicate that uridine rescue results in a relatively faster clearance of FUra from bone marrow RNA and tumor RNA and a marked increase in the rate of recovery of DNA synthesis only in the bone marrow.

Pyrimidine nucleoside kinases allow both normal and tumor cells to salvage circulating nucleosides such as uridine, dThd, or dUrd (3, 16, 22). The activity of uridine/cytidine kinase (EC 2.7.1.48), the rate-limiting enzyme for the utilization of uridine, varies in different tumor lines (24) and even, to some extent, within a series of human tumors (1, 2). Thus, even in a tumor where the predominant effect of FUra is due to FUra/RNA, differences in the utilization of uridine in tumor as compared to normal host tissues might allow for a selective antitumor effect.

The organ systems most susceptible to FUra toxicity are the gastrointestinal tract and the bone marrow (9). In order to see if uridine rescue from the lethal toxicity of FUra correlated with effects against a sensitive normal tissue, we monitored the effects of FUra on bone marrow nucleated cellularity. We showed that the ability of uridine to rescue mice from the lethal toxicity of FUra correlated with its sparing effect on the extent of the FUra-induced depression in bone marrow cell numbers, and, in addition, there was a more rapid recovery to pretreatment levels. Furthermore, the specificity of uridine, as compared to dThd or dUrd, to rescue mice from the lethal toxicity of FUra correlated with the protective effect on the FUra-induced depression of bone marrow cellularity which occurred with the uridine, but not dThd or dUrd, infusion. Martin *et al.* (20) reported that the depression in WBC of mice given a single dose of FUra was not as great after uridine rescue.

Our previous observations that uridine, but not dThd or dUrd, rescues mice from the lethal toxicity of FUra (14, 15) have now been extended to the related fluorinated pyrimidines, FUrd and FdUrd. Our studies with FUrd and FdUrd in normal mice suggest that, as with FUra, it may be possible to increase the therapeutic effectiveness of these antimetabolite nucleosides against tumor-bearing mice by the use of uridine rescue.

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